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IgM Antibody Detection of ppUL80a and ppUL32 by Immunoblotting: An Early Parameter for Recurrent Cytomegalovirus Infection in Renal Transplant Recipients

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The value of IgM detection for the early diagnosis of an active cytomegalovirus (CMV) infection in renal transplant recipients was evaluated prospectively. Sequential serum samples obtained from 22 allograft recipients with active CMV infection were tested for the presence of CMV-specific immunoglobulin M antibodies (IgM) by an enzyme-linked immunosorbent assay (ELISA) and a microparticle enzyme immunoassay (MEIA) and were compared with the Western-immunoblotting technique (IB). The time course of CMV IgM antibody detection was evaluated in relation to the shell vial assay (SVA), CMV disease, and immunosuppressive regimen.

By IB, IgM antibodies against the capsid protein ppUL80a and the basic matrix phosphoprotein ppUL32 were detected in all 22 recipients with active CMV infection. Using the MEIA and the ELISA, the presence of CMV IgM antibodies was detected in 17 (77%) and ten (46%) of these 22 recipients, respectively.

The SVA was the earliest parameter for detection of primary CMV infection in seven of nine (78%) recipients, in contrast to two of 13 (15%) patients with recurrent CMV infection ($P < .05$). The detection of IgM antibodies by IB was the earliest parameter for detection of recurrent CMV infection in seven out of 13 (54%) recipients in contrast to one out of nine (11%) patients with primary CMV infection ($P < .05$). During a primary CMV infection, the development of an abundant IgM antibody response was associated with recovery from CMV disease and the end of the viremic phase.

KEY WORDS: cytomegalovirus, IgM, immunoblotting, shell vial assay, transplant recipients

INTRODUCTION

In immunocompromised patients, cytomegalovirus (CMV) may cause life-threatening disease. Early diagnosis of CMV infection is, therefore, important for appropriate treatment. Virus culture methods in addition to detection of viral antigens or viral nucleic acids in clinical specimens are used frequently for diagnostic purposes [Bij et al., 1988a; Gleaves et al., 1985; Vlieger et al., 1992; Boland et al., 1993]. The detection of CMV-specific immunoglobulins, especially of class M (IgM), can provide supportive evidence for both the diagnosis of active CMV infection [Demmler et al., 1986; Smith and Shelley, 1988] and the prognosis of CMV disease [Landini, 1993; Pouteil-Noble et al., 1993]. IgM detection can be used as diagnostic parameter during both primary and recurrent infection [Griffiths et al., 1982, 1984; Bij et al., 1988b; Champsaur et al., 1988; Landini and Michelson, 1988]. However, its significance seems to be limited [Rasmussen et al., 1982; Stagno et al., 1985; Landini et al., 1986; Rautenberg et al., 1992], and the specificity and sensitivity of the different techniques are unclear [Lazzarotto et al., 1992; Kraat et al., 1993].

The aim of the present study was to evaluate CMV IgM antibody detection as a method for early diagnosis of an active CMV infection in allograft recipients and to compare the results with the shell vial assay. Therefore, a prospective study was carried out in which sequential serum samples obtained from allograft recipients were analyzed by three different serologic techniques. An enzyme-linked immunosorbent assay (ELISA) and a microparticle enzyme assay (MEIA) were compared with Western-(immuno)blotting technique (IB) using both purified structural viral proteins of the CMV AD169 strain as well as the major nonstructural DNA-binding recombinant protein (ppUL44). In addition, the time course of virologic parameters for diagnosis of CMV infection and the relation to CMV disease and immunosuppressive regimen were studied.

MATERIALS AND METHODS

Patients

Eighty-two patients receiving a renal allograft were monitored for CMV infection. The transplant recipients

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were divided into four subgroups according to the CMV serostatus of the donor (D) and recipient (R): seropositive donor, seropositive recipient (D+R+); seropositive donor, seronegative recipient (D+R−); seronegative donor, seropositive recipient (D−R+); and seronegative donor, seronegative recipient (D−R−). The immunosuppression regimen and treatment of acute rejection were as described previously [Kraat et al., 1994].

Definition of CMV Infection and CMV Disease

The criteria for an active CMV infection were based on one or more of the following items: detection of the virus in blood and/or urine, seroconversion of CMV antibodies, a significant (at least fourfold) increase of CMV antibody titer between acute and convalescent sera.

The diagnosis of CMV disease was as described previously [Metselaar, 1990], namely, illness with two of the following otherwise unexplainable symptoms: fever (>38°C, measured in the axilla) for at least 3 consecutive days, leukocytopenia ($<3 \times 10^9$/L), thrombocytopenia ($<100 \times 10^9$/L), liver abnormalities (>2.5 times the normal upper limit), gastrointestinal, lung, or central nervous system involvement confirmed by concomitant positive CMV culture or serology.

Antibody Detection and Virus Isolation

Blood samples for serology, heparinized whole blood, and urine specimens for detection of infectious virus were collected weekly during hospitalization, or monthly up to 1 year after transplant. Specimens were submitted more frequently when there was clinical suspicion of CMV infection. Sera were tested routinely for the presence of CMV antibodies by a latex agglutination test (CMV Scan, Becton Dickinson Microbiology Systems, Cockeysville, MD) and a standard complement fixation method [Lennette, 1992].

Routine tissue culture was carried out for the detection of CMV in leukocytes harvested from whole blood and urine, using human embryo fibroblast (HEF) monolayers. For the early detection of infectious virus the shell vial assay (SVA) was carried out 48 hr after inoculation, using the monoclonal antibody E13 directed against the immediate early antigen of CMV (Biosoft, Paris, France) [Griffiths et al., 1984; Gleaves et al., 1985].

Serological Tests

The immunoblotting technique (IB), using the CMV AD169 strain, was carried out essentially as described previously [Braun et al., 1983; Landini et al., 1985]. Sera were used routinely at a 1:10 dilution. A serum specimen was considered to contain CMV-specific IgM antibodies if one or more of the major structural viral proteins ppUL32 (150 kDa), ppUL75 (82 kDa), ppUL83 (65 kDa), ppUL55 (55 kDa), ppUL80a (38 kDa), or ppUL99 (28 kDa) were visible [Landini et al., 1985; Kraat et al., 1993; Landini and Spaete, 1993]. In addition, all the sera were tested by IB for reactivity to the major nonstructural DNA-binding recombinant protein of ppUL44 (52 kDa) [Ripalti et al., 1989, 1994].

The microparticle enzyme immunoassay (MEIA) (IMx CMV IgM antibody assay, Abbott Laboratories, Chicago, IL) and the enzyme-linked immunosorbent assay (ELISA) (Vironostika anti CMV IgM Micro Elisa System, Organon Technika, Boxtel, The Netherlands) were performed according to the instructions of the manufacturers. Sera were routinely used at a 1:100 dilution. For MEIA and ELISA IgM antibody assay index of all sera was calculated from the optical density (OD) of the test serum divided by the OD of the reference serum, supplied by the manufacturer. Serum samples with an index equal to or higher than 1 were considered to be positive for IgM antibodies.

Statistical Analysis

Data analysis was carried out using the Wilcoxon matched-pairs signed-ranks test. Differences with a P value of less than .05 were considered to be statistically significant. All data analyses were done with SPSS-pc programs.

RESULTS

Presence of IgM Antibodies

Twenty-two of 82 renal transplant recipients experienced active CMV infection (six D+R+, eight D+R−, seven D−R+, and one D−R−). One hundred forty-eight serum samples from these patients and 15 control sera from recipients without CMV infection were tested for the presence of CMV IgM antibodies by IB, MEIA, and ELISA. As shown in Figure 1, IgM antibodies reactive with the ppUL32 and ppUL80a proteins were found by IB in all 22 patients. IgM antibodies against the other proteins were detected less frequently. According to the serostatus of the recipient the IgM IB profiles showed considerable variation. As shown in Figure 2, in the R− group the mean (± SD) number of proteins reactive in the IB was significantly higher than in the R+ group, 6.0 ± 1.3 versus 3.7 ± 1.3, respectively ($P < .05$). In ten of the 22 (46%) recipients IgM antibodies directed against ppUL44 were found. In seven out of nine (78%) R− recipients, reactivity with ppUL44 was found, in contrast to three out of 13 (23%) R+ recipients ($P < .05$).

The number of patients with sera reactive by MEIA and ELISA varied. IgM antibodies were found by MEIA in 17 out of 22 (77%) recipients, whereas ten out of 22 (46%) recipients were reactive by ELISA. The mean (± SD) of highest IgM index of the R− and R+ recipients obtained by MEIA and ELISA are also shown in Figure 2; both techniques were significant higher in the R− recipients than in the R+ recipients (R−: MEIA 6.3 ± 1.3 and ELISA 7.2 ± 2.9; R+: MEIA 1.4 ± 1.1 and ELISA 0.8 ± 0.1; ($P < .05$).

The serum samples obtained from the recipients without CMV infection were nonreactive by all tests.

Time of Detection of Active CMV Infection

To analyze which detection method was the first positive parameter for demonstration of active CMV infection, we compared detection of infectious virus in blood and the detection of IgM antibodies. By timing of sample collection, the presence of virus in blood as well as IgM

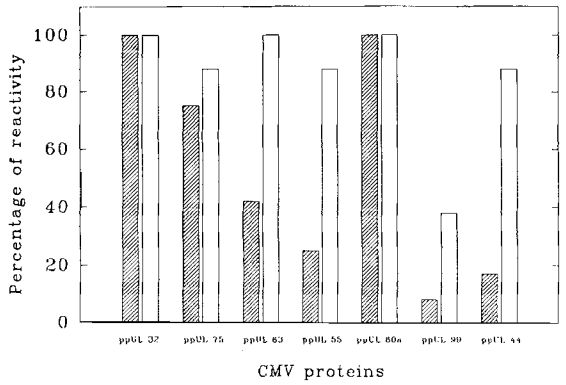

Fig. 1. The percentage of initially seropositive (hatched bars) and seronegative (open bars) recipients showing IgM antibody reactivity with CMV ppUL32 (150 kDa), ppUL75 (82 kDa), ppUL83 (65 kDa), ppUL55 (55 kDa), ppUL80a (38 kDa), or ppUL99 (28 kDa) as detected by IB.

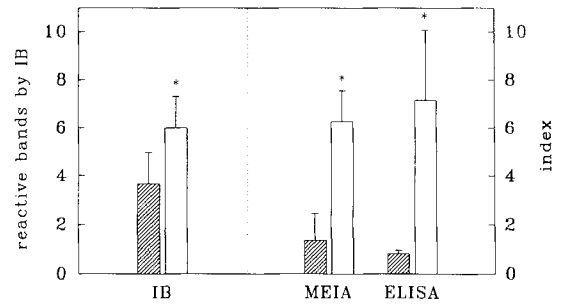

Fig. 2. The mean (± SD) number of reactive bands by IB and the mean (± SD) index by MEIA and ELISA in seropositive (hatched bars) and seronegative (open bars) recipients. *P value indicated above the bars are significant ($P < .05$).

antibody detection by IB were the earliest parameters for active CMV infection in 13 of 22 (59%) renal transplant recipients (Table I). The SVA was the first parameter in nine recipients, whereas the IB was the first in eight recipients. In seven out of nine R− recipients the SVA was the earliest detectable parameter, as compared to two out of 13 R+ recipients ($P < .05$).

In eight out of the 13 R+ recipients IgM antibodies were detectable first by IB, as compared to one out of the nine (11%) R− recipients ($P < .05$) The SVA was the first detected parameter for active infection in seven out of nine R− recipients and in only two out of 13 R+ recipients. The detection of viremia by SVA preceded the serum IgM antibody peak by 2.5 ± 2.9 days (mean ± SD) in the R− group. This is in contrast to the R+ group in which the CMV IgM antibody peak preceded the last detection by the SVA by 18 ± 41.3 days (ns).

Virologic and Clinical Surveillance After Transplantation

Symptomatic illness developed in nine of the 22 (41%) allograft recipients with active CMV infection. The presence of IgM antibodies was not related to the development of symptomatic CMV infection. CMV disease developed in five out of nine R− recipients and in four out of 13 R+ recipients. In six of the nine symptomatic allograft recipients, CMV disease waned at the moment the number of proteins reactive with serum IgM antibodies by IB was maximal. In addition, the index of IgM antibodies in MEIA and ELISA reached the highest detectable level at that time.

In this study no correlation was found between the immunosuppression regimen or acute rejection and treatment used, and the IgM antibody response in this patient group (data not shown).

The clinical symptoms of the CMV infection were monitored in all patients in association with the IgM response and virus culture. In general, in the R− recipients a high index of IgM response was found. A typical example of a D+R− patient is shown in Figure 3A. In this patient the SVA was the first parameter of active CMV infection and was positive at the time symptomatic disease developed, followed by detection of IgM by IB. Thereafter, a high IgM antibody index was found in both the MEIA and ELISA. The number of proteins reactive in the IB increased at the time the patient recovered from CMV disease. In the R+ patient group the IgM detection by IB preceded the detection of infectious virus. The clinical and virological course of one of the R+ patients is shown in Figure 3B (D+R+). This recipient did not meet the criteria for CMV disease. In this patient IgM antibodies directed against the 38- and 150-kDa proteins were detected first and were followed by virus detection in the SVA. Despite enduring virus isolation in subsequent leukocyte samples, serum samples showed low IgM index by the MEIA, but no reactivity by ELISA.

DISCUSSION

In this prospective study, 82 renal transplant recipients were monitored for CMV infection for at least 1 year after transplantation. The suitability of three CMV IgM detection assays for the early diagnosis of active CMV infection was tested and was compared with the shell vial assay. IgM antibodies were detected by MEIA, ELISA, and the IB technique.

CMV IgM antibodies were detected more frequently by IB than by MEIA or ELISA (100% vs. 77% and 46%, respectively). During recurrent CMV infections in particular, the MEIA and ELISA showed a low sensitivity in comparison to the IB. Possible explanations for this finding can be the low serum dilution used in the ELISA and MEIA and the absence of some structural viral proteins in the antigenic material of these assays. The IgM antibody response, as indicated by the index values in MEIA and ELISA, was significantly higher after primary infection than after recurrent infection. These high index values corresponded with the relatively large number of proteins reactive with IgM by IB. IgM antibodies against the ppUL32 and ppUL80a structural viral proteins could be detected in all 22 recipients with an active CMV infection by IB, indicating that these structural proteins are strong immunogens, irrespective of the pretransplant serostatus. These results are in agreement

TABLE I. First Parameter(s) for Active CMV Infection in Transplant Recipients, as Obtained by Timing of Sample Collection

First parameter				Patient group by CMV serostatus of the recipients	
	IgM antibodies detected by				
CMV viremia	IB	ELISA	MEIA	R+	R−
+	−	−	−	2/13[a]	7/9
−	+	−	−	7/13	1/9
+	+	−	−	3/13	0/9
−	+	−	+	1/13	0/9
+	+	+	+	0/13	1/9
13	13	1	2		

[a]No. of cases reactive in the appropriate test/total No. of renal transplant recipients in the group.

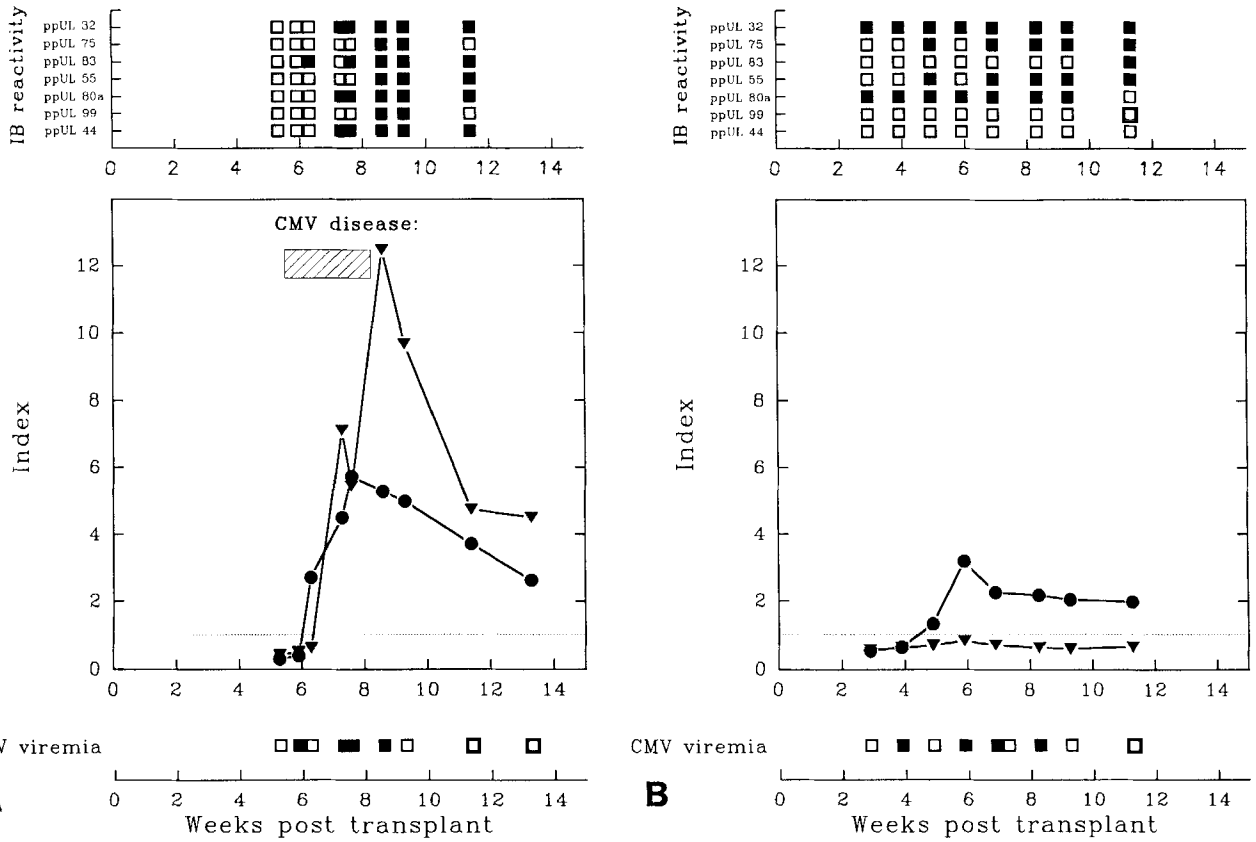

Fig. 3. Virological, serological, and clinical events in two renal transplant recipients with active CMV infection (**A:** primary infection, **B:** recurrent infection). The parameters were CMV viremia as detected by SVA (positive, solid squares; negative, open squares) and IgM antibody detection by IB (reactive protein, solid squares; non-reactive, open squares) and by MEIA (solid circles) and ELISA (solid triangles) index.The index is expressed as the ratio between the optical density of the test serum and the reference serum. An index of 1 or higher was considered to be reactive; the cut-off value of 1 is indicated by the dotted line. The period of symptomatic disease (hatched bar) was recorded.

with those reported in other studies [Landini and Michelson, 1988; Re and Landini, 1989; Landini et al., 1991; Fischer et al., 1993]. Another observation is that the serostatus of the recipient has an influence on the IgM response. In general, the IgM antibody titers measured by MEIA and ELISA and the number of proteins reactive by IB were significantly higher in the R− recipients than in the R+ recipients. This indicates that IgM antibody response is stronger during primary CMV infection and that IgM antibodies to more antigens are developed in this patient group. The only exception was the presence of antibodies against ppUL99, which could not be detected in all R− recipients. This observation corroborates earlier studies reporting a poor IgM response to ppUL99 [Landini et al., 1988]. IgM antibodies against the nonstructural recombinant protein ppUL44 were more frequently detected after primary than after recurrent CMV infection, a finding also reported by others

[Landini et al., 1989; Revello et al., 1991], suggesting that ppUL44 can be used as a marker for primary infection. Recently, some data show that ppUL44 is also a good marker for recurrent CMV infection [Vornhagen et al., 1994].

In our study, only 14 of the 22 (64%) recipients had IgM antibodies reactive with ppUL83. This observation is in contrast with the results obtained by others, in which this antigen is considered as one of the most immunogenic antigens for IgM antibodies [Landini et al., 1988, 1993; Gonczol et al., 1989; Zanten et al., 1993]. Possible explanations for the low number of recipients with IgM antibodies against ppUL83 in our study could be differences either in viral load in this patient population or the type of maintenance immunosuppressive regimen and rejection treatment used.

The finding of IgM detection by IB as an early indication of CMV infection in transplant recipients is confirmed by others [Ghisetti et al., 1995]. Another method used frequently for early detection of CMV infection is CMV antigenemia assay [Schirm et al., 1987; Bij et al., 1988b]. In general, this assay correlates well with active CMV infection after transplantation [Berg et al., 1989; Ehrnst et al., 1993]. However, other studies indicated that this test does not have advantages over the SVA and did not detect all viremias [Erice et al., 1992; Lipson et al., 1993]. Studies in our laboratory have shown that both tests, antigenemia and SVA, are equally sensitive for detection of active CMV infection in renal allograft recipients (unpublished results).

A highly sensitive method for the detection of active CMV infection is the polymerase chain reaction (PCR) undertaken on leukocytes or plasma. However, the disadvantage of this technique is false-positive results. In addition, the presence of viral nucleic acids in the peripheral blood can be detected irrespective of the development of disease and can persist for weeks or months after the acute infection has resolved [Gerna et al., 1991].

In R− recipients, active CMV infection was first detected by the SVA, whereas in R+ recipients IgM antibody detection by IB was the earliest parameter for active CMV infection. Overall, active CMV infection was detected later by the two commercially available IgM antibody detection methods (MEIA and ELISA) than by IB or the SVA.

The presence of IgM antibodies was not predictive of the development of symptomatic CMV disease, an observation that was also made in other studies [Marsano et al., 1990; Fischer et al., 1993]. In the present study, maximal IgM antibody reactivity corresponded with the end of the viremic period and with the disappearance of CMV disease after primary infection. In patients with recurrent CMV infection, the maximum index for IgM was lower and did not clearly correspond with the end of viremia. In these patients, the viremic period ended at 3 weeks after the IgM antibody level reached its maximal reactivity, in contrast to the early clearance of infectious virus after primary infection. In conclusion, both the SVA and IB were found to be suitable tests for early diagnosis of active CMV infection in renal transplant recipients. The SVA was most convenient for early detection of primary CMV infection. The detection of IgM antibodies against ppUL32 and ppUL80a by IB was most suitable for early diagnosis of recurrent CMV infection. In addition, a high level IgM response was shown to be a good indicator for recovery from CMV disease and is a marker for the end of the viremic period after primary infection.

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